DISTRIBUTION OF A TRYPSIN-LIKE PROTEINASE IN THE RAM SPERMATOZOON

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The trypsin-like proteinase (TLP) of rabbit spermatozoa (Stambaugh & Buckley, 1969) attracted interest following the discovery that trypsin inhibitors impede the penetration of spermatozoa through the zona pellucida (Stambaugh, Brackett & Mastroianni, 1969; Zaneveld, Robertson & Williams, 1970). Stambaugh & Buckley (1969) recovered most of this proteinase in an acrosomal fraction prepared by treating spermatozoa with the surfactant Hyamine 2389 (Hartree & Srivastava, 1965). Using fluorescein-labelled trypsin inhibitors, they established for several mammalian species that TLP is restricted to the acrosomal region of the sperm cell (Stambaugh & Buckley, 1970).

Difficulties arise when attempts are made to correlate this work with electron micrographic studies of the changes undergone by rabbit and rodent spermatozoa during egg penetration and with electron micrographs of spermatozoa treated with Hyamine. At the stage when a spermatozoon enters the zona pellucida, the acrosome has already undergone a degradation ('acrosome reaction') which results in the inner acrosomal membrane (i.a.m.) becoming the limiting membrane over the entire acrosomal region apart from its distal region, the equatorial segment (Bedford, 1968, 1971; Pikó, 1969). It has thus been assumed (Stambaugh & Buckley, 1970; Bedford, 1970; Zaneveld, Robertson, Kessler & Williams, 1971) that TLP, as a zona-penetrating enzyme, must be associated with the i.a.m. The standard treatment with Hyamine solubilizes most of the TLP of rabbit spermatozoa (Stambaugh & Buckley, 1969) and also, as we have found, of ram spermatozoa. Nevertheless, it is established for bull (O'Donnell, Symons & Wooding, 1970) and ram (Hartree, 1971) spermatozoa that such treatment leaves the i.a.m. attached to the sperm head. To obtain direct experimental evidence on the location of TLP in ram spermatozoa, we have developed a procedure for removing acrosomes which does not involve surfactants and which leaves the i.a.m. *in situ*.

The activity of TLP was measured at 25° C in a pH-stat with $N\alpha$ -benzoyl-L-arginine ethyl ester (BAEE) (Walsh & Wilcox, 1970). The assay system (1.6 ml) contained 2 mM-BAEE, 0.1 M-CaCl₂, 1.5 mM-tris, plus HCl to pH 8.2. Since ejaculated spermatozoa contain trypsin inhibitors, true measures of TLP content require that sperm fractions be acidified at 0° C to pH 2.5 to dissociate inhibitor-enzyme complexes (Zaneveld, Dragoje & Schumacher, 1972) before being added to buffered BAEE solution.

Ram semen (16 ml) was diluted with 4 vol. sucrose-citrate (0.275 M-sucrose,

16 mM-citric acid, NaOH to pH 6.5) and centrifuged at room temperature under conditions ($\sim 600\text{ g}$) that leave cytoplasmic droplets in the supernatant fraction (Allison & Hartree, 1970). Sperm pellets must be loose enough to resuspend when the capped tubes are rocked gently. The cells were washed once with sucrose-citrate and once with 0.25 M-sucrose. The washed spermatozoa were resuspended in 0.25 M-sucrose and stored at -20°C . To detach acrosomes, the frozen suspension was thawed, diluted with 0.25 M-sucrose to ten times original semen volume (o.s.v.) and stored at 20 to 22°C for 1 hr. Subsequent steps were effected at 0 to 5°C . The suspension was agitated, in 10-ml portions, with a whirlimixer (vortex mixer), this treatment being timed

Table 1. Fractionation of washed, whirlmixed spermatozoa (W) into denuded spermatozoa and two wash-fluids containing acrosomal material, followed by solubilization at pH 3 of trypsin-like proteinase (TLP) in the denuded sperm fraction

Exps	Fraction	Full TLP activity (U) Relative values		Net (inhibited) TLP activity (U)	Cell count corrected for dilution from o.s.v. ($10^{-9} \times \text{cells/ml}$)
1	W	75	87	33	2.5
	Wash-fluid 1	7.3		0.5	
	Wash-fluid 2	4.7		3.5	
	Denuded sperm.	74	100	70	2.4
	Solubilized TLP	83	98	84	
2	W	68	77	20	2.4
	Wash-fluid 1	4.4		2.5	
	Wash-fluid 2	4.6		2.6	
	Denuded sperm.	78	100	68	2.0
	Solubilized TLP	88	101	85	

Units of activity (U) are μmol BAEE hydrolysed/min/vol. of fraction derivable from 1 ml semen. To obtain full activities, samples were stored at 0°C and pH 2.5 for 4 hr and added to BAEE in CaCl_2 -tris buffer. To obtain net activities, BAEE was added to fractions previously diluted with buffer. o.s.v.—original volume of ram semen.

(20 to 30 sec) to produce at least 50% of 'denuded' spermatozoa, i.e. spermatozoa that, under phase contrast, were recognizably without acrosomes. A sample of this suspension (W) was kept for analysis. The remainder was centrifuged (20 min, 500 g), the cells were resuspended in sucrose, whirlmixed as before and centrifuged again. The spermatozoa, now virtually all denuded, were stored in 0.25 M-sucrose. The two supernatant wash-fluids, containing acrosomes and small particles, were kept. In electron micrographs, the denuded spermatozoa closely resemble published micrographs of Hyamine-treated spermatozoa (O'Donnell *et al.*, 1970; Hartree, 1971), i.e. the plasma membrane is missing and over the entire acrosomal region (apart from the equatorial segment) the limiting membrane is the i.a.m.

To extract TLP from denuded spermatozoa, the suspension was stirred at 0°C , cautiously acidified with N-HCl to pH 3.0 and centrifuged for 15 min at $20,000\text{ g}$. A second extraction with 0.25 M-sucrose pH 3 ($2 \times \text{o.s.v.}$) left an enzymatically inactive residue. The combined acid extracts contained TLP in soluble form which was stable for several weeks at 5°C .

Table 1 summarizes our results. The ram semen contained $\sim 3 \times 10^9$ cells/ml;

thus the recovery of denuded cells was about 70%. Losses occurred mainly through loose packing of sperm pellets during the washing of spermatozoa. Tighter packing improved recovery but damaged the cells. The combined activities of denuded spermatozoa and wash-fluids always exceeded the activity of W from which the three fractions were derived. This may have been due in part to incomplete removal of acrosomes in W. The wash-fluids and W contained considerable amounts of TLP inhibitor: however, removal of inhibitor in the wash-fluids left the denuded spermatozoa almost free of inhibitor. From the full activity values in Table 1, it is clear that TLP was retained by ram spermatozoa during denudation. (Preliminary experiments indicate that the same holds for rabbit and bull spermatozoa.) Since TLP is restricted to the acrosome (including the equatorial segment—Stambaugh & Buckley, 1970), we conclude that it is specifically associated with the i.a.m. although its distribution within the segment remains uncertain.

When denuded spermatozoa are added to the standard assay system, TLP is rapidly solubilized. If, in the assay system, CaCl_2 is replaced by 0.25 M-sucrose, no solubilization occurs but, again, the activity of denuded spermatozoa is nearly as high as that of the acid extract. We therefore deduce that after the acrosome reaction occurs, the TLP bound to the i.a.m. is readily accessible to extracellular substrates.

Among marine invertebrates, lysins diffusing from spermatozoa can attack egg-membranes in advance of contact between the gametes (Wada, Collier & Dan, 1956; Colwin & Colwin, 1960). Passage of a mammalian spermatozoon through the zona pellucida, however, is a result of the cutting of a narrow hole with a cross-section no greater than the widest cross-section of the sperm head (Dziuk & Dickmann, 1965; Yanagimachi, 1966; Bedford, 1970). This is consistent with the expected effects of a surface-bound sperm proteinase upon the zona substance. Bedford (1970) has raised the possibility that the zona-penetrating enzyme may be beneath the i.a.m., i.e. in the perinuclear space. We find that the TLP in whirlimixed spermatozoa is at least 98% sedimentable at 100,000 g. This strongly suggests that any TLP beneath the i.a.m. is unlikely to diffuse through that membrane to become functional during zona penetration. There are reasons, however, for supposing that enzymes in spermatozoa, and especially in the equatorial segment, play a part in the fusion of spermatozoon with vitellus and perhaps in activation of the egg (Bedford, 1970; Hartree, 1971).

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